Monomeric alcohol oxidase is preferentially digested by a novel protease from Candida boidinii

Stewart, Mary Q.; Dijk, Ralf van; Veenhuis, Marten; Goodman, Joel M.

Published in:
Biochimica et Biophysica Acta-Molecular Cell Research

DOI:
10.1016/S0167-4889(01)00176-8

Publication date:
2002

ELSEVIER

Biochimica et Biophysica Acta 1542 (2002) 160–172

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Monomeric alcohol oxidase is preferentially digested by a novel protease from *Candida boidinii*

Mary Q. Stewart [a], Ralf van Dijk [b], Marten Veenhuis [b], Joel M. Goodman [a,*]

[a] *Department of Pharmacology, University of Texas Southwestern Medical School, 5323 Harry Hines Boulevard, Dallas, TX 75390-9041, USA*

[b] *Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 Haren, The Netherlands*

Received 29 August 2001; received in revised form 31 October 2001; accepted 2 November 2001

Abstract

A protease activity has been partially purified from peroxisomal matrix fractions of the methylotrophic yeast *Candida boidinii*. The enzyme migrates as a single peak on a sucrose velocity gradient with an apparent native molecular mass of ~80–90 kDa. Activity can be recovered from nonreducing sodium dodecyl sulfate gels as a ~20 kDa species, suggesting it is an oligomer. The protein exhibits chymotrypsin-like activity and cleaves the model compound suc-L-L-V-Y-AMC. Additionally, monomers of alcohol oxidase (AO), an abundant protein of *C. boidinii* peroxisomes, generated in vitro or in pulse-radiolabeled cells, are preferentially sensitive to degradation by the protease. Sensitivity is lost over time in vivo as AO folds and matures into octamers, suggesting that the protease may be involved in these processes.

Keywords: Peroxisome; Microbody; Serine protease; Alcohol oxidase; *Candida boidinii*

1. Introduction

Peroxisomes are ubiquitous organelles that are comprised of a matrix surrounded by a single membrane and are essential for synthesis and degradation of lipids and other compounds [1]. Peroxisomes have no genome; all peroxisomal proteins are nuclear encoded and must be imported from the cytoplasm.

There are two well characterized targeting motifs that direct proteins from the cytosol to the peroxisomal matrix [2,3]. Most matrix proteins have a conserved targeting motif, termed PTS1, at their extreme carboxy termini. A few matrix proteins have an amino-terminal PTS2 sequence that is cleaved in some species upon or shortly after translocation across the membrane. Cognate receptors bind matrix proteins in the cytosol and transport them to a docking complex on the peroxisomal surface.

The mechanism of entry of protein cargo into the matrix following docking is obscure. Unlike some import mechanisms, proteins need not be unfolded to cross the peroxisomal membrane. Several proteins are known to act after the docking step but their precise roles are unknown [4]. Our group and others have shown that folded proteins and oligomers can enter peroxisomes [5]. There is no evidence that unfolding occurs during this process; even colloidal

* Corresponding author. Fax: +1-214-648-2994.
E-mail address: joel.goodman@utsouthwestern.edu (J.M. Goodman).

0167-4889/02/$ – see front matter
PII: S0167-4889(01)00176-8

gold particles can enter the organelles [6]. Furthermore, the association of receptors and cargo may not be interrupted until the complex enters the matrix [7,8].

Molecular chaperones can bind to peroxisomal proteins in the cytosol, and they are also found on the peroxisomal surface [9,10]. Whether they help fold these precursors or keep them unfolded and competent for import is not clear. No members of classical chaperone families have been demonstrated to exist in the peroxisomal matrix with the possible exception of peroxisomes in plants [11], suggesting that the bulk of protein folding occurs before translocation into the matrix.

Alcohol oxidase (AO), a flavin-containing octamer, is the only peroxisomal matrix protein known to be imported as a monomer and to oligomerize within the matrix [12,13]. It is a very abundant protein of methylotrophic yeasts when grown on methanol as the carbon source. Flavin auxotrophs have been generated to study the binding of this cofactor to AO as it relates to peroxisomal import. Low external flavin concentrations result in a decrease in AO import and in aberrant AO aggregates both in the cytosol and in the peroxisomal matrix [14]. This defect cannot be attributed to a decrease in cellular energy stores. One cannot definitively conclude that AO binds flavin after translocation but it is clear that the absence of the cofactor results in decreased AO import.

While it is unknown whether or not peroxisomes have the capacity to fold proteins within the matrix, peroxisomes can catalyze the proteolytic maturation of precursors. Three steps of peroxisomal protein maturation have been attributable to peroxisomal activities: the clipping of the PTS2 amino terminal signal, the further degradation of the PTS2 peptide, and the scission of the A subunit of acyl-CoA oxidase to generate B and C subunits [15–17]. The degradation of the PTS2 peptide may be catalyzed by a peroxisomal protease formerly known as the insulin-degrading enzyme [15]. The cleavage of the PTS2 peptide from the primary translation product occurs in association with a preperoxisomal vesicle, at least in the yeast *Yarrowia lipolytica* [18]. Several different endoprotease activities have also been identified and characterized in plant peroxisomes although no sequences are available [19].

In this report we identify, enzymatically characterize, and partially purify a novel protease activity from the peroxisomal fraction of *Candida boidinii*. We find that it is highly active against newly synthesized AO monomers, but not mature octamers. This protease sensitivity reveals differences in AO folding during import and assembly, in which this protease may be involved.

2. Experimental procedures

2.1. In vitro transcription/translation

In vitro labeled proteins were synthesized with the T3 or T7 promoters from Bluescript plasmids containing the appropriate genes (Stratagene Cloning Systems, La Jolla, CA), using the TNT Coupled Reticulocyte Lysate System (Promega Corp., Madison, WI) with [^{35}S]methionine (translation grade, New England Nuclear Life Science Products, Boston, MA). Synthesis was allowed to proceed for 90 min. Plasmid constructs encoding ornithine decarboxylase and gamma-glutamyl cysteine synthetase were the kind gift of Meg Phillips (UT Southwestern Medical Center, Dallas, TX).

2.2. Protease activity assay using ^{35}S-labeled in vitro translated alcohol oxidase

Standard in vitro protease reactions consisted of 5 μl of translated product (see above) incubated with enzyme samples in a final volume of 20 μl for 30 min unless otherwise indicated. Sample buffer [20] was immediately added and the sample boiled prior to performing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Gels were then treated with 1 M sodium salicylate (pH 5.5) for 5 min, dried, and exposed to film.

2.3. Peroxisomal matrix purification

Peroxisomal matrix from highly purified organelles was obtained from a 10-l culture of methanol-grown *C. boidinii* as previously described [21]. Assays for cytochrome *c* oxidase [22] and catalase [23] were used to verify organellar separation. The peroxisomal fraction was diluted with 2 volumes of 30% sucrose

and centrifuged at 31 000×*g* for 45 min at 2°C in a JA17 rotor (Beckman Coulter, Fullerton, CA). The pellet was resuspended in 10 ml TE buffer (50 mM Tris–Cl (pH 8.5), 5 mM EDTA), which lysed the peroxisomes. Membranes were removed by centrifuging the peroxisomal lysate at 100 000×*g* for 15 min at 2°C in a TLA 100.3 rotor (Beckman Coulter).

2.4. Fluorogenic peptide assay

Peptides conjugated with 7-amino-4-methylcoumarin (AMC), (Bachem, Torrance, CA), were utilized for analysis of proteolytic activity. The rate of AMC release was monitored for 5 min with the Photon Technology International Fluorometer and Felix software (Lawrenceville, NJ) with excitation and emission wavelengths set to 370 and 440 nm, respectively. Rates were linear over the measurement. A unit of activity is defined as the rate of increase of fluorescence in photons detected per second over the 5-min assay.

Peroxisomal matrix was used for the measurements of peptide specificity. One-ml reactions in TE containing 50 μM of peptide and 10 μl of 2 mg/ml matrix in TE were assayed. Reactions with chymotrypsin (Sigma, St. Louis, MO) were identical except they contained 0.00055 U of enzyme instead of matrix.

For inhibitor studies, matrix (9 mg) in TE was applied to a 35 ml 5–20% continuous sucrose gradient, which was centrifuged at 28 000 rpm in a SW-28 rotor (Beckman Coulter) for 48 h. Peak fractions containing protease activity were pooled and concentrated for use as the source of enzyme. Inhibitors were preincubated with 5 μl (430 ng protein) of pooled fraction for 5 min at concentrations shown. The peptide substrate suc-Leu-Leu-Val-Tyr-AMC was then added to a final concentration of 30 μM in 1 ml final volume in TE. Approximate IC_{50}s were calculated using Sigma Plot (SPSS, Chicago, IL).

2.5. Denaturation/renaturation of protease

Partially purified protease from a sucrose velocity gradient as described above was incubated with nonreducing sample buffer (50 mM Tris–Cl (pH 6.8), 10% glycerol, 1% SDS), at either 25°C or 50°C for 30 min. The two samples were electrophoresed through a 9% SDS polyacrylamide gel at 75 V. Each lane was then cut into 0.5-cm slices, noting relative position to prestained molecular mass markers. Renaturation was performed by incubating each section in 1 ml of TE buffer containing 0.25% Triton X-100 on a rocker overnight. Gel sections were washed with 1 ml of TE buffer, and then each slice was crushed into a slurry in 20 μl of TE buffer to extract the protein. An additional 30 μl of TE buffer was added before the eluted material was assayed for activity against in vitro translated AO product.

2.6. Column chromatography

Econo-Pac prepacked 1-ml columns and Macro-prep resins were purchased from Bio-Rad (Hercules, CA). Columns were run as per manufacturer's instructions. Econo-Pac columns were utilized to develop a large scale purification protocol. Purification was then performed using Macro-Prep resin for larger columns. Purified peroxisomes were lysed in Tris buffer (pH 8.5) and matrix obtained as described above, except without EDTA. Matrix was loaded onto a 15 ml Macro-prep HiQ column that had been pre-equilibrated with 50 mM Tris–Cl buffer, pH 8.5. Fractions were collected during elution with an increasing salt gradient to 500 mM NaCl in 50 mM Tris–Cl buffer, pH 8.5. Protease activity of the fractions was assessed by the fluorogenic peptide assay (above), and active fractions were pooled. Ammonium sulfate was added to the pooled fractions to a final concentration of 1.6 M for loading onto a 15 ml Macro-prep methyl-HIC column, which had been equilibrated with 50 mM Tris–Cl (pH 8.5), 250 mM NaCl, and 1.6 M ammonium sulfate. Fractions were collected from a step gradient from high to low salt. Each step consisted of 25 ml of 1.6 M, 1.2 M, 0.8 M, 0.4 M, and 0 ammonium sulfate. Each fraction was tested by peptide assay for protease activity. Active fractions were pooled and concentrated using a Microcon concentrator (Millipore Corp., Bedford, MA) for use in gel filtration analysis. Gel filtration was done using Ultrogel AcA34 resin (IBF Biotechnics, France). Gel filtration was performed in 50 mM Tris–Cl (pH 8.5) buffer containing 250 mM NaCl. Fractions were collected and analyzed as before.

2.7. pH release assay

Peroxisomes were harvested from a sucrose gradient as described [21]. Eight separate microfuge tubes, each containing 200 μl of the peroxisomal fraction, were centrifuged at 20 000×*g* for 20 min at 4°C. The supernatant was aspirated off, and the pellets resuspended in 100 μl of one of eight different pH buffers: 5.5, 6.0, 6.5 (each in 50 mM MES), 6.8, 7.4, 8.0, 8.5 and 9.2 (each in 50 mM Tris–Cl). The tubes were spun as before and the supernatant removed to a separate tube. The pellet was resuspended in 100 μl of the corresponding buffer. The supernatant and pellet fractions were assayed for catalase [23], alcohol oxidase [24], and protease activity (peptide assay).

2.8. Reagents and other methods

Growth in methanol, conversion to spheroplasts, and in vivo labeling of *C. boidinii* cells was performed as described [12]. Culturing of cells in D-alanine, oleate, and glucose has been described previously [25]. Protein concentrations were determined using a commercial protein assay reagent (Bio-Rad) Protease inhibitor cocktail ('Complete Cocktail') was purchased from Boehringer (Mannheim, Germany) and is generally effective against serine-, cysteine-, and metallo-proteases. The cocktail was prepared as a 50× stock solution and used at a final concentration of 25× unless otherwise noted. SDS–PAGE [20] was performed with 9% gels and a separating gel at pH 9.2. Chymotrypsin and individual protease inhibitors were purchased from Sigma (St. Louis, MO), except for AEBSF (Calbiochem-Novabiochem Corp., San Diego, CA), and aprotinin and pepstatin A (Boehringer). Phenyl Sepharose, purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

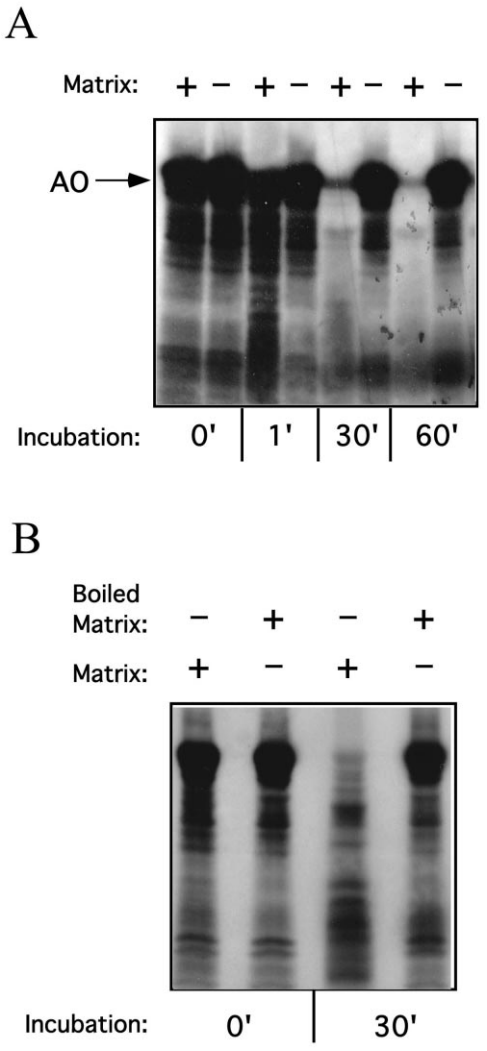

Fig. 1. (A) ^{35}S-Labeled in vitro translated alcohol oxidase was incubated with purified peroxisomal matrix (0.5 mg/ml final concentration) or buffer at 30°C for times indicated. Sample buffer was added and the samples were boiled immediately, then subjected to SDS–PAGE and fluorography. (B) ^{35}S-Labeled in vitro generated alcohol oxidase was incubated for times indicated with matrix (0.5 mg/ml final concentration) or matrix which had been boiled for 5 min.

3. Results

Alcohol oxidase (AO) is imported into peroxisomes as monomers, where it assembles into mature octamers [12]. In vitro synthesized AO was analyzed by sucrose velocity sedimentation and found to be monomeric (data not shown). The AO generated was unable to spontaneously assemble, even with the components contained in the reticulocyte lysate. This suggested that factors necessary for assembly were likely to be in the matrix where oligomerization occurs in vivo. To try to mimic assembly in vitro, monomeric AO was incubated with matrix from purified peroxisomes from *C. boidinii* at 30°C. We found that AO was degraded in the reaction; significant degradation was apparent after 1 min, and very little was left after 30 min (Fig. 1A). A commercial

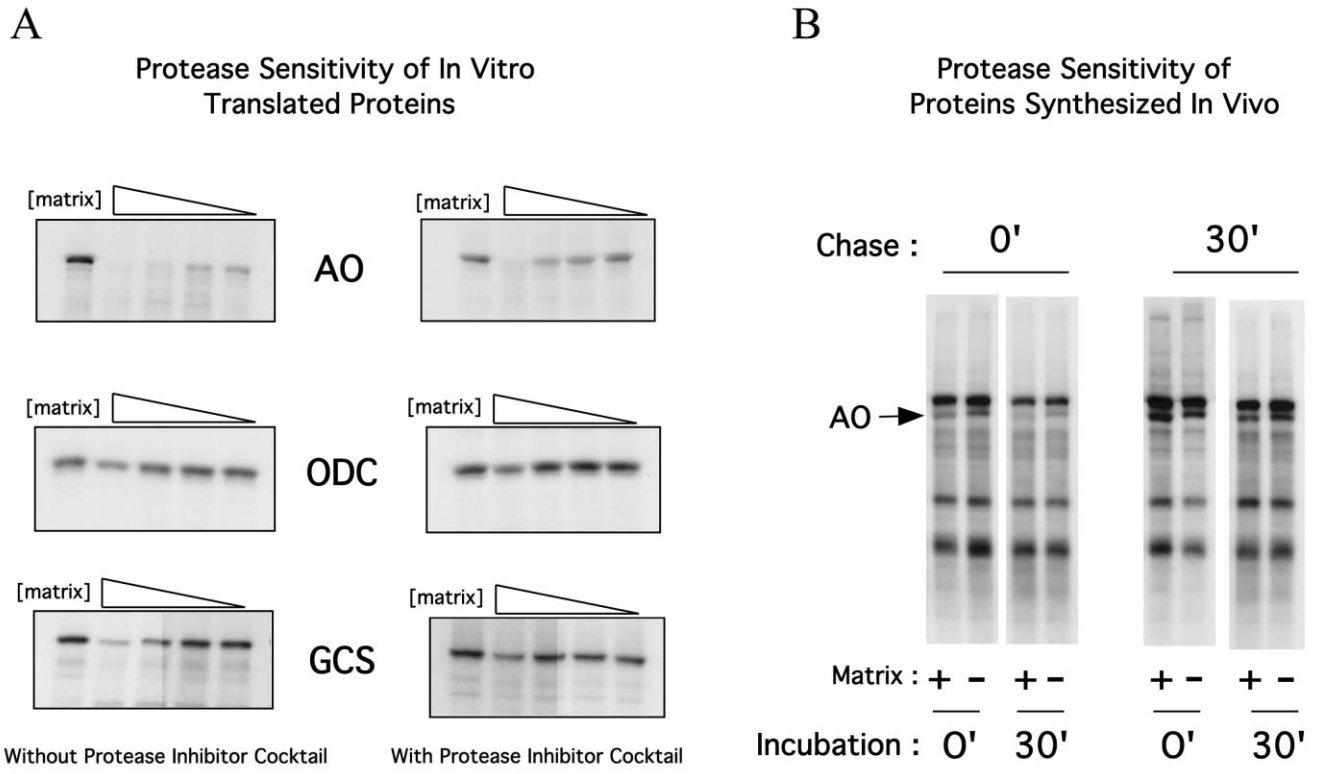

Fig. 2. Substrate sensitivity to protease. (A) The indicated proteins were translated in vitro and subjected to dilutions of concentrated purified matrix (0, 5, 2.5, 1.25, and 0.625 mg/ml final concentrations, respectively) both in the presence and absence of 'Complete (protease inhibitor) Cocktail'. ODC, ornithine decarboxylase; GCS, γ-glutamylcysteine synthase. (B) Spheroplasts were labeled with [^{35}S]methionine for 5 min then chased with cold methionine for 0 or 30 min as indicated. Cells (100 μl from each chase time point) were centrifuged at 5000×*g*. The pelleted cells were lysed in 200 μl 50 mM Tris–Cl (pH 8.5), 5 mM EDTA, and protease inhibitor cocktail. Concentrated matrix (125 μg/ml final concentration) or buffer was then incubated with 5 μl of lysate from each chase time in a total volume of 20 μl at 30°C for either 0 or 30 min. Sample buffer was added, and samples were boiled immediately and subjected to SDS–PAGE.

protease inhibitor cocktail, consisting of pepstatin A, aprotinin, leupeptin, and AEBSF, had only a moderate effect on proteolysis of AO, even at 30 times the recommended concentration (data not shown). However, degradation did not occur after boiling the matrix (Fig. 1B).

To examine the specificity of the protease activity against other proteins, we began by comparing in vitro substrates. Two nonperoxisomal substrates, ornithine decarboxylase (ODC) and γ-glutamylcysteine synthetase (GCS) as well as AO, were synthesized in vitro and subjected to the matrix protease at various concentrations. AO was much more sensitive to proteolysis than either ODC or GCS (Fig. 2A, left). At 0.625 mg/ml matrix protein, for example, ODC and GCS were not affected, while only 30% of AO remained (determined by densitometry). A cocktail of protease inhibitors (at ten times the recommended concentration) had only a slight effect on activity (Fig. 2A, right). Control experiments demonstrated that trypsin degraded all in vitro products equally, and that the inhibitor cocktail almost completely blocked the trypsin degradation (data not shown). Thus, the preferential degradation of AO by the matrix protease reflects more than the general unfolded state of the in vitro products.

To further analyze the substrate specificity of the protease we incubated a radiolabeled yeast lysate with purified matrix. Immediately following a 5-min pulse and cell disruption, a 30-min incubation with matrix resulted in loss of most of the radiolabeled AO, while there was little discernible degradation of other proteins in the lysate (Fig. 2B). However, AO was resistant to proteolysis after a 30 min in vivo

chase, presumably reflecting its folding and conversion to octamer [24]. In summary, monomeric AO, synthesized in vitro and in vivo is very sensitive to the protease compared with other observed proteins.

Matrix was subjected to sucrose velocity sedimentation to determine the approximate size of the native activity. Fractions were then assayed against in vitro synthesized AO as substrate. The activity migrated as a single peak corresponding to a molecular mass (assuming a globular shape) of ~80–90 kDa (Fig. 3A). We found that we could recover enzyme activity from nonreducing SDS gels. When matrix was incubated with Laemmli sample buffer (without reducing agent) at 25°C before electrophoresis, some activity could be recovered from a gel slice corresponding to 72–135 kDa (note activity against AO in the second lane of the upper panel of Fig. 3B), consistent with its native size on sucrose gradients. Interestingly, heating the matrix in nonreducing sample buffer at 50°C resulted in recovery of activity in a gel slice corresponding to ~20–23 kDa (Fig. 3B). The reason for the apparent increase in activity is unknown. These results suggest that the protease is an ~80–90 kDa oligomer with the active subunit corresponding to about 20 kDa.

The activity of the matrix protease against model peptides was then tested to examine the site specificity of the activity. Peptides conjugated to 7-amino-4-methylcoumarin (AMC) were used to allow monitoring of activity by fluorescence upon release of the chromophore. Fig. 4A shows that the proteolytic activity is very low against H-Val-Leu-Lys-AMC. Significant activity is present against H-Val-Val-Arg-AMC. However, the matrix showed robust activity against suc-Leu-Leu-Val-Tyr-AMC, suggesting a chymotrypsin-like activity. A comparison with chymotrypsin against tyrosine-containing peptides is shown in Fig. 4B; activity is normalized to the suc-Leu-Leu-Val-Tyr-AMC peptide. While neither enzyme cleaves the short H-Tyr-AMC peptide, chymotrypsin is more active against the H-Leu-Val-Tyr-AMC peptide. However, the activity of the matrix protease against suc-Leu-Leu-Val-Tyr-AMC peptide suggests that it is in the chymotrypsin family.

Using the suc-Leu-Leu-Val-Tyr-AMC peptide cleavage as an indicator of activity, we examined the effect of a range of inhibitors on the protease. In order to examine inhibition, we first determined

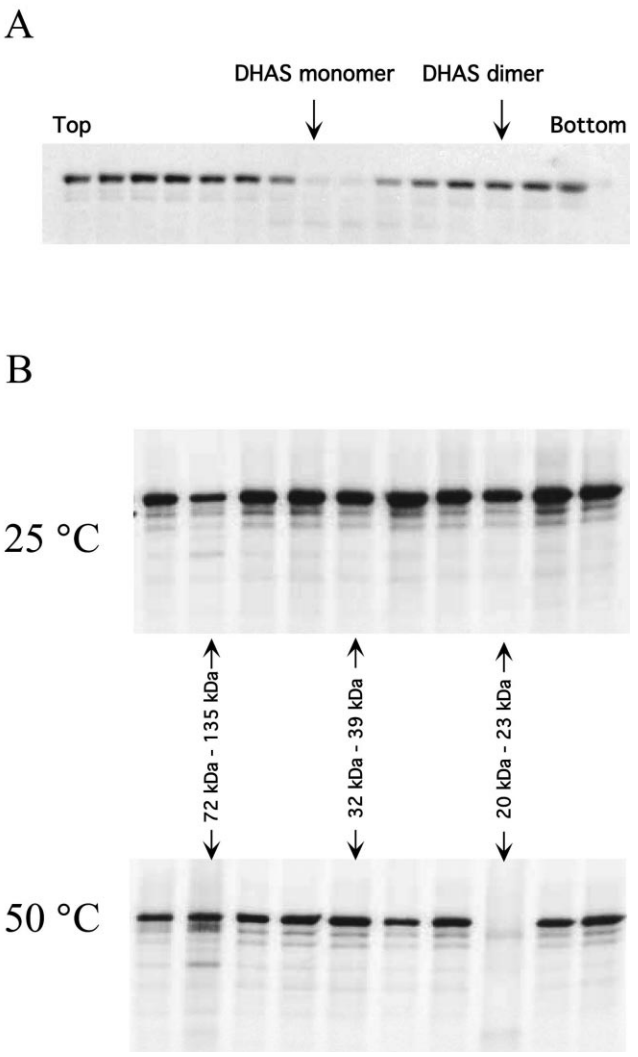

Fig. 3. (A) Protease activity, utilizing in vitro AO as substrate, of fractions from a 5–20% sucrose gradient (centrifuged in a Beckman SW60 rotor at 60 000 rpm for 12 h at 4°C). Arrows indicate position of radiolabeled DHAS monomer (78 kDa) and dimer (156 kDa) on a gradient centrifuged under identical conditions. (B) Sucrose gradient peak material was subjected to denaturation at the temperatures indicated before SDS–PAGE under nonreducing conditions. The gel lanes were divided into horizontal slices, which were then incubated to renature the protease, extracted, and analyzed against in vitro synthesized AO. Molecular mass ranges of the appropriate slices are indicated.

the apparent affinity of the peptide substrate. Matrix protease, partially purified from a sucrose gradient similar to that illustrated in Fig. 3A, was used to determine the apparent K_m of the enzyme against suc-Leu-Leu-Val-Tyr-AMC. As shown in Fig. 4C, the apparent K_m is 34 μM. For all the inhibitor

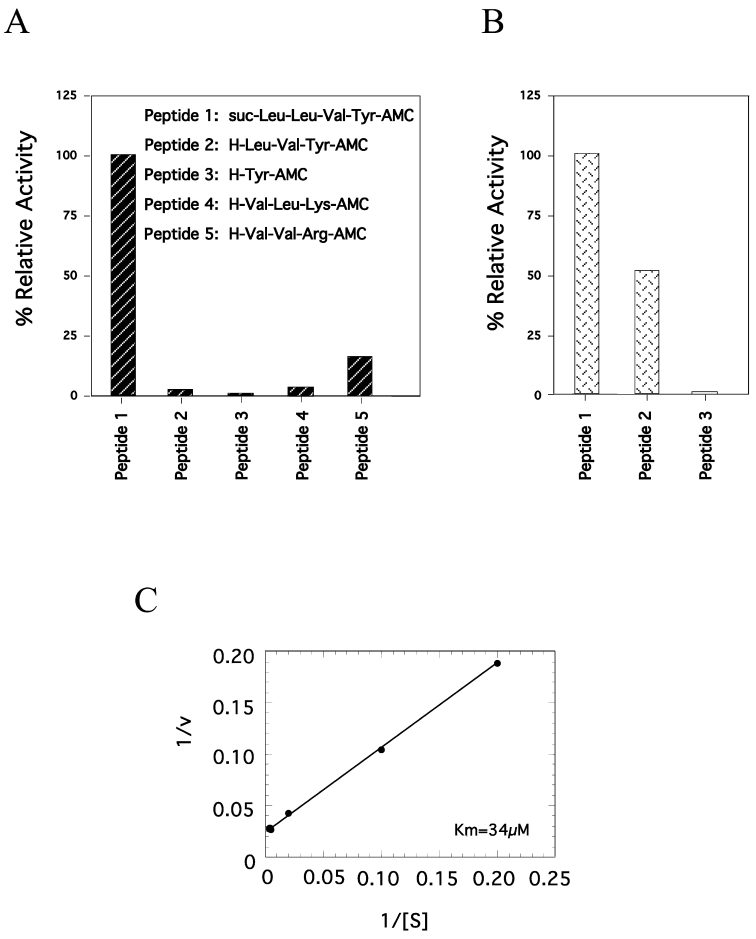

Fig. 4. Substrate preference of matrix protease. (A) Each peptide substrate was subjected to the addition of purified matrix; the activity against peptide 1 was set at 100% activity. (B) Peptides 1–3 were also exposed to chymotrypsin under the same conditions. The activity for chymotrypsin against peptide 1 was set at 100% activity. The absolute rate of hydrolysis of peptide 1 by chymotrypsin was 2.7 times that of matrix. (C) Determination of the apparent K_m of matrix enzyme against suc-Leu-Leu-Val-Tyr-AMC peptide. Sucrose gradient peak fraction was used. [S] in μM; *v*, rate in fluorescence units per second.

studies, a peptide substrate concentration of 30 μM was used to maximize sensitivity of the assay. An inhibitor study is shown in Table 1. Several serine protease inhibitors, PMSF, antipain, chymostatin and leupeptin were active against the matrix protease, while AEBSF, benzamidine, and aprotinin were not. In contrast, the aspartyl protease inhibitor, pepstatin A, and the metallo-protease inhibitors, EDTA and *o*-phenanthroline, did not affect activity. These data confirm that the matrix activity is that of a serine protease.

To further purify the matrix protease, we subjected the matrix fraction to column chromatography using the peptide assay to monitor activity. As an initial study, the protease was subjected to purification on 1-ml columns that separated proteins based on anion exchange (HiQ), cation exchange (HiS), and hydrophobic interactions (HIC) or other properties (ceramic hydroxyapatite type II (CHT-II)). Purified matrix was loaded onto each. Reasonable increases in specific activity (6–36-fold) were obtained for all columns, with the HiQ column having the best recovery of activity (55%).

Based on these results, we chose to first purify the protease on a preparative HiQ column. The resulting profile is illustrated in Fig. 5A. Fractions 7–13 were then pooled and applied to a hydrophobic HIC column (Fig. 5B). Interestingly, these profiles, especially that from the HIC column, show an inhomogeneity of protease activity. This may reflect partial dissoci-

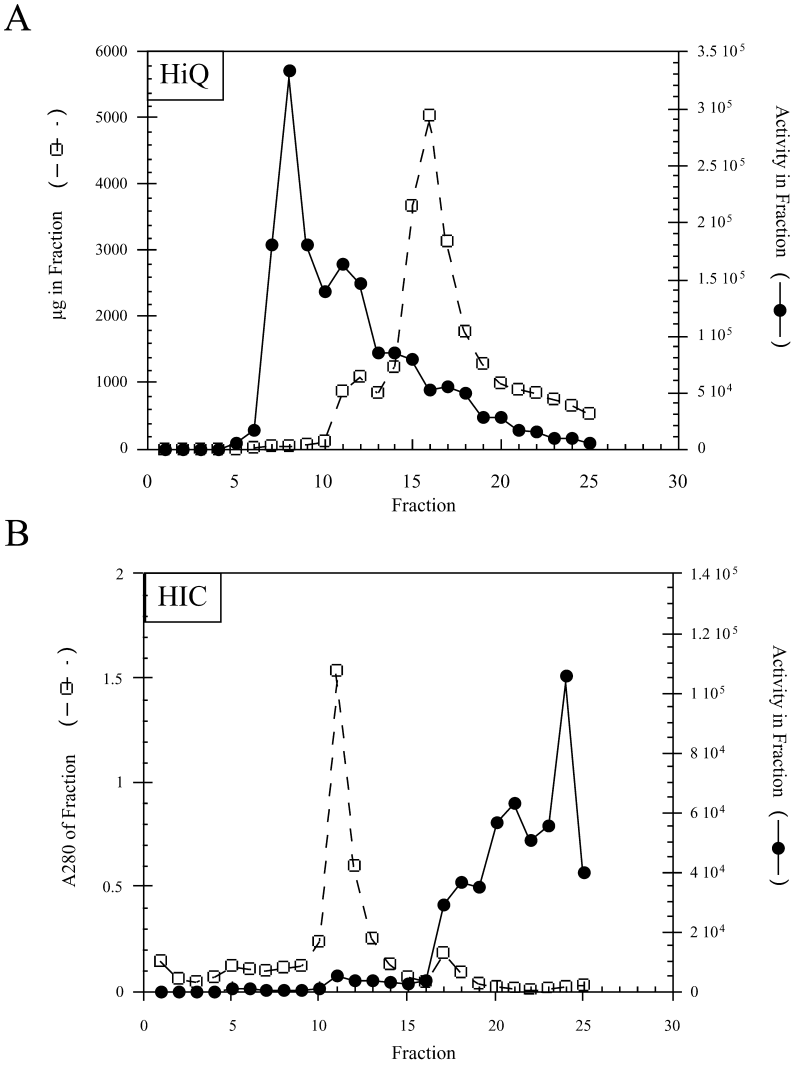

Fig. 5. Chromatographs of protease purification steps. (A) Profile of protein and protease activity from the HiQ column 35 mg of matrix was applied. (B) Profile of protein and protease activity from the HIC column. 3.8 µg of protein pooled from fractions 7–13 of the HiQ column was applied.

ation of subunits during purification or the presence of more than one activity. Table 2 summarizes the results of purification through these two columns. A 2500-fold purification of activity was achieved with 27% recovery. Unfortunately, we could make no further progress beyond this point due to unacceptable losses of activity on subsequent columns. This may reflect subunit destabilization in the dilute protein environment of the column or loss of a critical factor. We could also not yet identity a candidate protease by inspection of stained gels of peak column fractions, although protein bands were very light even by silver strain. The specific activity of the protease against the suc-Leu-Leu-Val-Tyr-AMC peptide after chromatography on HiQ and HIC columns was only 6–8-fold lower than pure chymotrypsin, suggesting that one additional step of purification would be sufficient if the enzyme were more stable (unpublished data).

Our source for the protease was purified peroxisomal matrix. To confirm that the activity was actually in peroxisomes, we compared the migration of organelles in a sucrose isopycnic gradient to the migration of protease activity. The starting material was a $25\,000 \times g$ organellar pellet, which is enriched in peroxisomes and mitochondria, obtained by differ-

Table 1
Effect of protease inhibitors on matrix enzyme

Inhibitor	Final concentration (M)	Relative activity (%)[a]	Approximate IC_{50}
PMSF	1×10^{-5}	77	50 μM
	5×10^{-5}	56	
	1×10^{-4}	27	
Antipain	5×10^{-9}	75	10 nM
	1×10^{-8}	46	
	1×10^{-6}	1	
Chymostatin	3×10^{-7}	59	400 nM
	5×10^{-7}	44	
	8×10^{-7}	34	
Leupeptin	2×10^{-5}	67	40 μM
	4.3×10^{-5}	45	
AEBSF	2×10^{-3}	97	>2 mM
Benzamidine	1×10^{-4}	96	>100 μM
	2×10^{-4}	92	
Aprotinin	1.5×10^{-6}	98	>1.5 μM
Pepstatin A	1.5×10^{-5}	100	>15 μM
EDTA	5×10^{-3}	99	>5 mM
o-Phenanthroline	1×10^{-3}	100	>1 mM

[a]Compared to absence of inhibitor, set at 100%.

ential sedimentation of a cell lysate [24]. In this subsequent sucrose gradient, mitochondria migrate to the middle of the gradient, and peroxisomes to the bottom (Fig. 6A). As expected, protease activity, as assayed both against AO monomers and synthetic peptide substrate, comigrated with peroxisomes (Fig. 6B,C, respectively). Very little activity was found elsewhere in the gradient.

To further verify the peroxisomal location of the protease, we made use of the observation that peroxisomes lyse at neutral pH in vitro, while they are stable at slightly acidic pH [24]. The release of two matrix proteins, catalase and AO, as a function of pH, can be easily measured (Fig. 7A,B, respectively). We compared these data to the release of protease from intact organelles (Fig. 7C). As expected, recovery of protease in the pellet fraction decreased at increasing pH. Surprisingly, this activity did not appear in the supernatant. These data can be explained by general instability of the protease since a pH profile of the matrix protease yielded a peak at pH of 8.0–8.5 (data not shown), similar to most serine proteases. The data suggests other factors exist within the organelle that regulates protease activity.

Finally, we compared the organelle and protease profiles in sucrose gradients from cells grown in different carbon sources. The matrix protease was derived from cells grown in methanol, which induces alcohol oxidase and the peroxisomal methanol assimilation pathway. When *C. boidinii* is grown on glucose, peroxisomes are very small and scarce, while cells cultured on oleate or D-alanine yield peroxi-

Table 2
Partial purification of protease

Column	Total activity (Δ/s)	Recovery of activity (%)[a]	Specific activity (Δ/s mg)	Fold purification[b]
Matrix	1.6×10^{6}	–	46 714	–
HiQ	1.2×10^{6}	75	394 38	8.45
HIC	4.4×10^{5}	27	116 916 579	2500

[a]Percentage of activity in matrix.
[b]Matrix set at 1.

somes of specific densities and functions specific for assimilating those carbon sources; the methanol pathway is not activated in cells under these conditions [25]. The profiles from organellar sucrose gradients are shown in Fig. 8. Again, the protease in cells grown on methanol very closely corresponded to that of catalase. Protease activity was seen on the gradients onto which organelles from the other carbon sources were applied, but there was less correspondence of protease activity with peroxisomes. Protease activity always appeared in peroxisomal fractions, but was elsewhere in the gradient as well. These profiles suggest that the expression or local-

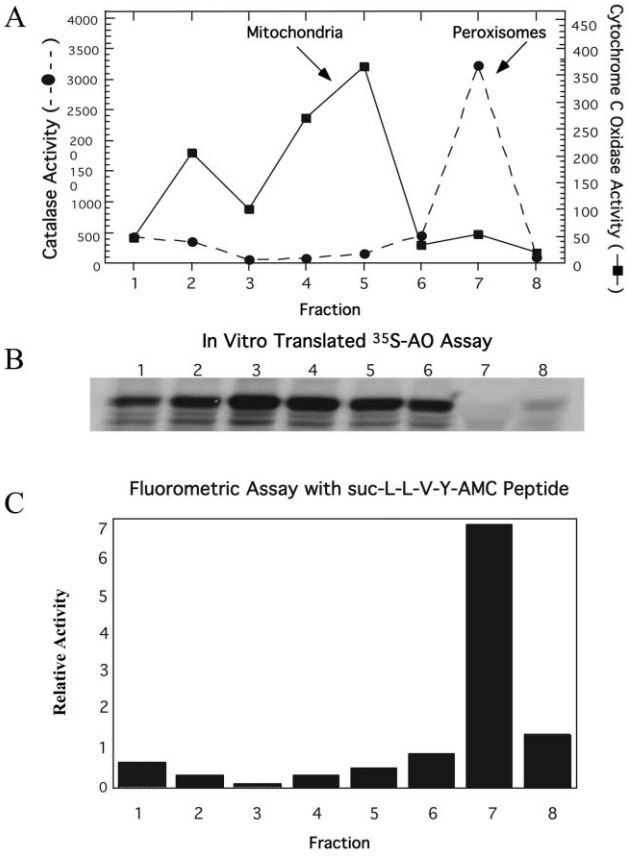

Fig. 6. Analysis of a discontinuous sucrose gradient used for peroxisomal purification. The gradient was fractionated into eight fractions from top to bottom. An equal percentage of each fraction was used to analyze activities across the gradient. (A) Catalase and cytochrome *c* oxidase activities across the sucrose fractions (●, catalase activity: ■, cytochrome *c* oxidase activity). (B) Degradation of in vitro translated AO by sucrose gradient fractions. (C) Proteolytic activity against 10 μM of the sucrose gradient fractions.

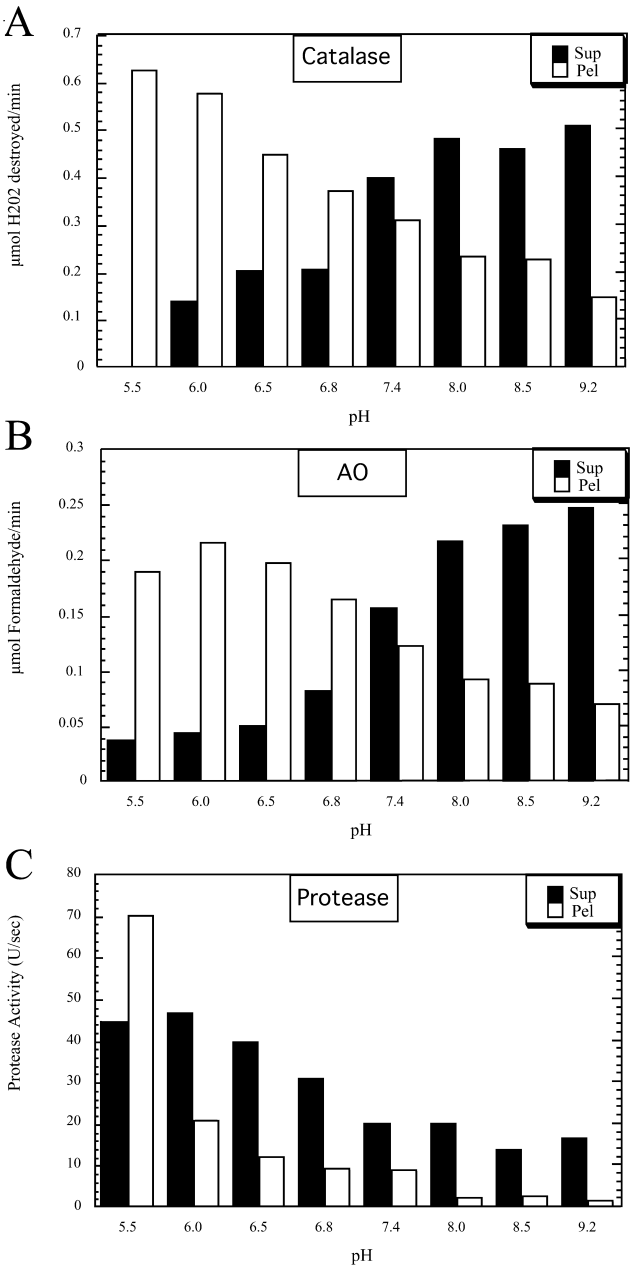

Fig. 7. pH release analysis of catalase activity (A), alcohol oxidase activity (B), and protease activity (by peptide assay) (C).

ization (or both) of organellar proteases is highly dependent on growth conditions. We conclude from our localization data that it is likely that we are studying a peroxisomal protease but that it may be specific for methanol-grown cells. The complex pattern of organellar proteases illustrated in Fig. 8 does not allow us to rule out contamination by an activity in other organelles.

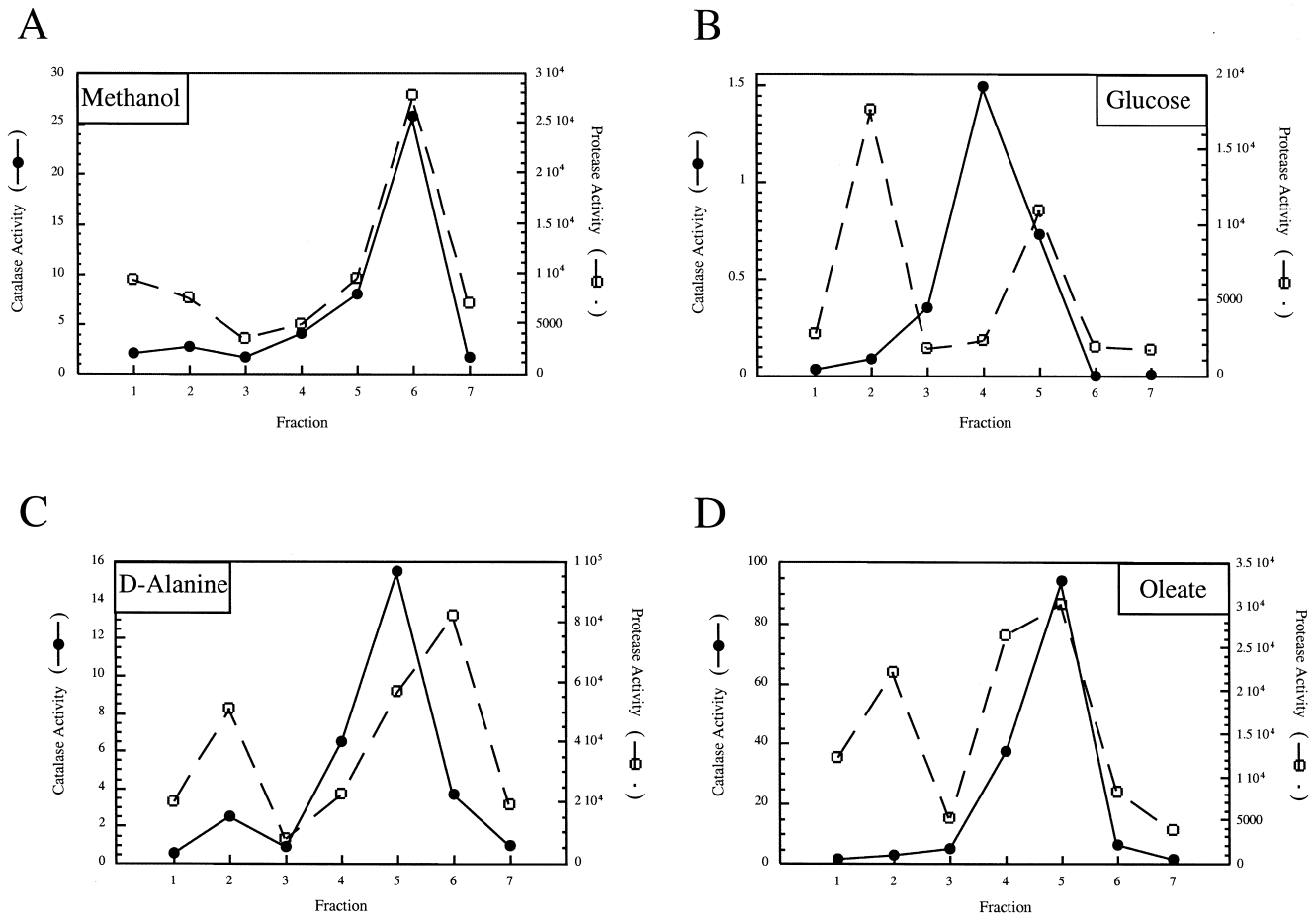

Fig. 8. Catalase (μmol H_2O_2 destroyed/min) and protease (against suc-Leu-Leu-Val-Tyr-AMC) activities were determined from fractions across discontinuous sucrose gradients of organelles from methanol (A), glucose (B), D-alanine (C), and oleate (D) grown *C. boidinii*.

4. Discussion

We describe a serine protease activity found in purified peroxisomal fractions in the methylotrophic yeast, *C. boidinii*. The protease behaves as an ~80–90 kDa oligomeric protein which can dissociate to yield a ~20 kDa active subunit. We show that monomeric alcohol oxidase is particularly sensitive compared to other new synthesized proteins in vitro and in vivo.

Several peroxisomal protease activities have been previously described. The IDE protease, while localized to peroxisomes, appears to function as a targeting signal protease for the PTS2 signal of thiolase and perhaps others [15]. Protease activities from plant peroxisomes, including a few serine proteases, have also been seen. It is suggested that these proteases catalyze protein turnover within the peroxisome during different stages of plant development [19]. None of the characteristics of these proteases or those of other known proteases match the activity described in our report. The single peak of protease activity on sucrose gradients suggests only one major activity. The activity described here appears to be specific towards AO (Fig. 2B), so we do not believe it is involved in general protein turnover.

Although some serine protease inhibitors block the enzyme, others do not. We noticed that the most active inhibitors contain a benzene ring in their structure, suggesting that this motif may be important in substrate recognition. In fact, a preliminary attempt to purify the enzyme with phenyl-Sepharose suggested that active protease could not be released from the column under standard conditions (data

not shown). The surprising result that PMSF but not AEBSF inhibited the protease further argues that the phenyl group (present in PMSF but not in AEBSF) is important for enzyme binding. Our protease is most active against the fluorogenic peptide, suc-Leu-Leu-Val-Tyr-AMC, additional evidence of structural specificity.

The protease retains activity after SDS–PAGE. Upon heating to 50°C in SDS before electrophoresis, however, it migrates as a ~20 kDa protein, indicating that the native enzyme must be a larger oligomer. Dissociation occurs in the absence of reducing agents, indicating that the subunits are not connected by disulfide bonds. The ~20 kDa form is more active against AO than the ~80 kDa species (Fig. 3B). This may reflect the ease at which the smaller form is extracted from the gel slice after electrophoresis or a preferential stability against SDS. More interestingly, the small form may be associated with a regulatory subunit that modulates its activity.

Partial purification of the protease also suggested that the protease is oligomeric. Both columns contained multiple peaks of activity (Fig. 5), which may be due to the disassociation of the oligomer. We were unable to confirm the sizes of the peaks by subsequent gel filtration, so it is unclear if the activity is made up of homogeneous activities or if multiple proteases are involved. Analysis of active fractions by silver stain did not provide any protease candidates although we were hampered by low amounts of protein.

The apparent specificity of the protease toward a peroxisomal protein suggests that the activity itself is associated with peroxisomes. Indeed, the protease colocalizes with catalase across a sucrose gradient from methanol-grown cells (Figs. 6 and 8A). Electron micrographs of the peroxisomal fraction have shown the fraction to be pure [24], so it is unlikely that the activity is from a contaminating organelle. Examination of α-mannosidase in *C. boidini*, normally a membrane-bound vacuolar marker in other yeast [26], revealed the mannosidase activity to be soluble and not associated with a vacuolar membrane (data not shown). The lack of suitable organellar makers led us to examine the protease activity in organelles from cells grown in other carbon sources where peroxisomes float to different sucrose densities. While the lack of colocalization of the protease with catalase in glucose and D-alanine suggests that the activity is not associated with peroxisomes, it may also indicate that different proteases are induced under these conditions, both peroxisomal and non-peroxisomal, and that the peroxisomal protease described in this report is only needed under conditions when large amounts of AO is expressed, as in methanol-grown cells. Further experiments to characterize the organellar proteases from cells grown on different carbon or nitrogen sources should yield information on whether different enzymes are induced under these conditions.

The peroxisome may require a mechanism to dispose of any protein that is improperly folded. This may be the role of the protease described here. The matrix of peroxisomes from methylotrophic yeasts is composed almost exclusively (by mass) of two proteins: AO and dihydroxyacetone synthase (DHAS); each comprises nearly 50% of matrix protein. DHAS converts to a dimer, its final oligomeric state, very quickly after synthesis in the cytosol [12,21] and is imported as a dimer. AO, on the other hand, remains in the cytosol for several minutes prior to its import. It may normally fold in the cytosol over this time. The unfolded AO may be a major threat to the organelle if it causes aggregation or instability of other proteins. Such a threat could be contained by the matrix protease. Whether the protease fulfills this mission is the subject of present research.

Acknowledgements

We would like to thank the members of the Goodman laboratory for many insightful discussions. We also would like to thank Shary N. Shelton for her able technical assistance. Additional thanks to the laboratory of Meg Phillips for providing plasmids utilized for in vitro expression studies and use of the fluorometer. We would also like to acknowledge Barbara Barylko for her suggestions in regards to the denaturation/renaturation assay. The authors gratefully acknowledge funding from NIH Grant GM-31859, the Robert A. Welch Foundation Grant I-1085, the Department of Pharmacology, UTSW, and also by a grant of the Netherlands Technology Foundation (STW), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

References

[1] C. Masters, D. Crane, The Peroxisome: A Vital Organelle, Cambridge University Press, Cambridge, 1995.
[2] E.H. Hettema, B. Distel, H.F. Tabak, Biochim. Biophys. Acta 1451 (1999) 17–34.
[3] S. Subramani, A. Koller, W.B. Snyder, Annu. Rev. Biochem. 69 (2000) 399–418.
[4] S. Subramani, Physiol. Rev. 78 (1998) 171–188.
[5] J.A. McNew, J.M. Goodman, Trends Biochem. Sci. 21 (1996) 54–58.
[6] P.A. Walton, P.E. Hill, S. Subramani, Mol. Biol. Cell 6 (1995) 675–683.
[7] V. Dammai, S. Subramani, Cell 105 (2001) 187–196.
[8] I.J. van der Klei, R.E. Hilbrands, J.A. Kiel, S.W. Rasmussen, J.M. Cregg, M. Veenhuis, EMBO J. 17 (1998) 3608–3618.
[9] W. Crookes, L. Olsen, J. Biol. Chem. 273 (1998) 17236–17242.
[10] P. Walton, M. Wendland, S. Subramani, R. Rachubinski, W. Welch, J. Cell Biol. 125 (1994) 1037–1046.
[11] B. Wimmer, F. Lottspeich, I. van der Klei, M. Veenhuis, C. Gietl, Proc. Natl. Acad. Sci. USA 94 (1994) 13624–13629.
[12] M.Q. Stewart, R.D. Esposito, J. Gowani, J.M. Goodman, J. Cell Sci. 114 (2001) 2863–2868.
[13] H.R. Waterham, K.A. Russell, Y. Vries, J.M. Cregg, J. Cell Biol. 139 (1997) 1419–1431.
[14] M.E. Evers, V. Titorenko, W. Harder, I. van der Klei, M. Veenhuis, Yeast 12 (1996) 917–923.
[15] F. Authier, J.J. Bergeron, W.J. Ou, R.A. Rachubinski, B.I. Posner, P.A. Walton, Proc. Natl. Acad. Sci. USA 92 (1995) 3859–3863.
[16] T. Osumi, M. Hijikata, N. Ishii, S. Miyazawa, T. Hashimoto, in: H. Fahimi, H. Sies (Eds.), Peroxisomes in Biology and Medicine, Springer, Berlin, 1987, pp. 105–114.
[17] B. Swinkels, S. Gould, A. Bodnar, R. Rachubinski, S. Subramani, EMBO J. 10 (1991) 3255–3262.
[18] V.I. Titorenko, H. Chan, R.A. Rachubinski, J. Cell Biol. 148 (2000) 29–44.
[19] S. Distefano, J. Palma, M. Gomez, L. Rio, Biochem. J. 327 (pt. 2) (1997) 399–405.
[20] U.K. Laemmli, Nature 227 (1970) 680–685.
[21] J.M. Goodman, J. Biol. Chem. 260 (1985) 7108–7113.
[22] N.E. Tolbert, Methods Enzymol. 31 (1974) 734–746.
[23] H. Lück, in: H.-U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Academic Press, New York, 1963, pp. 885–894.
[24] J.M. Goodman, C.W. Scott, P.N. Donahue, J.P. Atherton, J. Biol. Chem. 259 (1984) 8485–8493.
[25] J.M. Goodman, S.B. Trapp, H. Hwang, M. Veenhuis, J. Cell Sci. 97 (1990) 193–204.
[26] T. Yoshihisa, Y. Ohsumi, Y. Anraku, J. Biol. Chem. 263 (1988) 5158–5163.